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INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
KARA JAMES ROBERT	CALHOUN SWARTZ	STANFORD, CALIFORNIA STANFORD, CALIFORNIA

☐ *Additional inventors are being named on the separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

IMPROVED METHODS OF IN VITRO PROTEIN SYNTHESIS

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☒ Customer Number 24353 →

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Place Customer Number Bar Code Label here

☐ Firm *or* Individual Name	Bozicevic, Field & Francis LLP				
Address	200 Middlefield Road, Suite 200				
Address					
City	Menlo Park	State	California	Zip	94025
Country	U.S.A.	Telephone	(650) 327-3400	Fax	(650) 327-3231

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Respectfully submitted,

SIGNATURE Pamela Sherwood

Date 11/20/2003

REGISTRATION NO. 36,677 *(if appropriate)*

TYPED or PRINTED NAME Pamela J. Sherwood

Docket Number: STAN-337PRV

TELEPHONE (650) 327-3400

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IMPROVED METHODS OF *IN VITRO* PROTEIN SYNTHESIS

BACKGROUND OF THE INVENTION

[01] The directed synthesis of biological macromolecules is one of the great achievements of biochemistry. With the advent of recombinant DNA (rDNA) technology, it has become possible to harness the catalytic machinery of the cell to produce a desired protein. This can be achieved within the cellular environment or *in vitro* using extracts derived from cells.

[02] Cell-free protein synthesis offers several advantages over conventional, *in vivo,* protein expression methods. Cell-free systems can direct most, if not all, of the metabolic resources of the cell towards the exclusive production of one protein. Moreover, the lack of a cell wall and membrane components *in vitro* is advantageous since it allows for control of the synthesis environment. For example, tRNA levels can be changed to reflect the codon usage of genes being expressed. The redox potential, pH, or ionic strength can also be altered with greater flexibility than *in vivo* since we are not concerned about cell growth or viability. Furthermore, direct recovery of purified, properly folded protein products can be easily achieved.

[03] *In vitro* translation is also recognized for its ability to incorporate unnatural and isotope-labeled amino acids as well as its capability to produce proteins that are unstable, insoluble, or cytotoxic *in vivo.* In addition, cell-free protein synthesis may play a role in revolutionizing protein engineering and proteomic screening technologies. The cell-free method bypasses the laborious processes required for cloning and transforming cells for the expression of new gene products *in vivo,* and is becoming a platform technology for this field.

[04] Despite all of the promising features of cell-free protein synthesis, its practical use and large-scale implementation has been limited by several obstacles. Paramount among these are short reaction times and low protein production rates, which lead to poor yields of protein synthesis and excessive reagent cost. Additionally, expensive reagents are required, and conventional methods are inefficient in the use of these expensive reagents.

[05] Particularly useful reactions combine *in vitro* transcription and translation, thereby providing a direct link between a DNA coding sequence and the protein product. However, the additional requirement for reagents to produce mRNA add to the overall cost of the reaction. Recent publications have discussed many different strategies for cost reduction of *in vitro* transcription reactions, including reusing DNA templates and employing fed batch protocols. For example, see Kern and Davis (1997) "Application of Solution Equilibrium Analysis to in -Vitro RNA Transcription" Biotechnology Progress 13:747-756; Kern and Davis

(1999) "Application of a Fed-Batch System to Produce RNA by In-Vitro Transcription" Biotechnology Progress **15**:174-184.

[06] Improvements are required to optimize *in vitro* transcription/translation systems. The continuous removal of the inhibitory by-product(s) as well as the continuous supply of substrates for synthesis may enable continuous or semicontinuous reaction systems to support synthesis over long reaction periods. However, these approaches may also result in inefficient use of substrates and therefore in high costs. Elucidation of inhibitory products, and prevention of their synthesis is of great interest for development of *in vitro* synthetic systems. Also important is the reduction of reagent costs. With present technology, the major reagent costs include the source of chemical energy, enzymes, DNA template, and NTPs. Methods of decreasing these costs while enhancing yield are of great interest.

Relevant literature

[07] US Patent 6,337,191 B1, Swartz *et al.* Kim and Swartz (2000) Biotechnol Prog. **16**:385-390; Kim and Swartz (2000) Biotechnol Lett. **22**:1537-1542; Kim and Choi (2000) J Biotechnol. **84**:27-32; Kim *et al.* (1996) Eur J Biochem. **239**: 881-886; Kim and Swartz (2001) Biotechnol Bioeng **74**:309-316; Davanloo et al., Proc Nat'l Acad Sci USA **81**:2035-2039 (1984); Datsenko et al., Proc Nat'l Acad Sci USA 97:6640-6645 (2000); Jewett et al. (2002) Prokaryotic systems for *in vitro* expression, in *Gene Cloning and Expression Technologies* (Weiner, M.P. and Lu, Q.: eds.), Eaton Publishing, Westborough, MA., pp. 391-411; Spirin et al., Science **242**:1162-1164 (1988).

[08] Cunningham and Ofengand (1990) Biotechniques **9**:713-714 suggest that adding inorganic pyrophosphatase results in larger reaction yields by hydrolyzing the pyrophosphate that accumulates. Pyrophosphate is inhibitory because the pyrophosphate complexes with the free magnesium ions leaving less available for the transcription reaction.

[09] Breckenridge and Davis (2000) Biotechnology Bioengineering **69**:679-687 suggest that RNA can be produced by transcription from DNA templates immobilized on solid supports such as agarose beads, with yields comparable to traditional solution-phase transcription. The advantage of immobilized DNA is that the templates can be recovered from the reaction and reused in multiple rounds, eliminating unnecessary disposal and significantly reducing the cost of the DNA template.

[10] U.S. Patent no. 6,337,191 describes *in vitro* protein synthesis using glycolytic intermediates as an energy source; and U.S. Patent no. 6,168,931 describes enhanced *in vitro* synthesis of biological macromolecules using a novel ATP regeneration system.

SUMMARY OF THE INVENTION

[11] Improved methods are provided for the *in vitro* synthesis of biological molecules, providing for improved yields, lowered costs, and enhanced utility. Improved cell-free protein synthesis reactions utilize glucose or pyruvate as an energy source for ATP production. Nucleoside triphosphates may also be replaced with nucleoside monophosphates. These improvements drastically decrease costs and increase the robustness of cell-free protein synthesis reactions. The reaction is substantially improved by the addition of exogenous phosphate.

BRIEF DESCRIPTION OF THE DRAWINGS

[12] Figure 1 is a bar graph comparing the level of *in vitro* protein synthesis with the component system of the present invention using pyruvate as the energy source and nucleoside monophosphates plus phosphate at various concentrations. CAT expression was determined from ^{14}C-leucine incorporation. Error bars represent the range from two separate experiments.

[13] Figure 2 is a bar graph comparing the level of *in vitro* protein synthesis with the component system of the present invention using glucose as an energy source with nucleoside triphophosphates and varying amounts of phosphate. CAT expression was determined from ^{14}C-leucine incorporation. Error bars represent the standard deviation from three separate experiments.

[14] Figure 3 is a bar graph comparing the level of *in vitro* protein synthesis with the component system of the present invention using glucose as an energy source and various components for successful cell-free reactions. CAT expression was determined from ^{14}C-leucine incorporation. Error bars represent the standard deviation from three separate experiments.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[15] Improved methods are provided for the *in vitro* synthesis of biological macromolecules, providing for improved yields, lowered costs, and enhanced utility.

Improved yield and lowered cost is obtained by a combination of reaction conditions, which include, without limitation, the use of glucose or pyruvate as an energy source, and the replacement of nucleoside triphosphates with nucleoside monophosphates, in the presence of exogenous phosphate.

[16] ATP is required for protein synthesis in cell-free reactions. Traditionally, a compound with a high-energy phosphate bond, such as phosphoenolpyruvate (PEP), is added to the reaction for this purpose. However, since glycolytic enzymes are also present in the cell extract, glucose can be used to drive cell-free reactions at a much lower cost and with a higher potential for ATP generation utilizing methods that also activate oxidative phosphorylation. The reaction conditions that generate this more natural environment result from providing a combination of the factors, as described below. This system is capable of significant protein production for up to 6 hours in an *in vitro* batch reaction. By mimicking the cellular environment, an enhanced synthetic capability is provided.

[17] In preferred embodiments of the invention, a reaction mixture as described herein is used for *in vitro* synthesis of biological macromolecules by using glucose or pyruvate as an energy source and by replacing nucleoside triphosphates with nucleoside monophosphates.

[18] Improved yields are provided by the addition of exogenous phosphate. Usually phosphate is provided at a concentration of at least about 1 mM, preferably at least about 5 mM, and not more than about 40 mM, usually not more than about 25 mM, and preferably at about 10 mM. Useful sources of phosphate (PO_4) include a variety of salts and acids that are compatible with the biological reactions, *e.g.* potassium phosphate, magnesium phosphate, ammonium phosphate, *etc.*

[19] The reactions are substantially free of polyethylene glycol. Performing synthesis in the substantial absence of polyethylene glycol allows for activation of oxidative phosphorylation and provides for improved folding; and may further be combined with, for example, the methods described in U.S. Patent no. 6,548,276, herein incorporated by reference.

[20] The methods of the present invention allow for production of proteins with the addition of an energy source to supplement synthesis, where the energy source is a phosphate free glycolytic intermediate, *e.g.* pyruvate, and glucose. The energy source may be supplied in concentrations of at least about 10 mM, of at least about 20 mM, more usually at least about 30 mM. Such compounds are not usually added in concentrations greater than about 250 mM, more usually not greater than about 150 mM. Additional amounts of the energy source

may be added to the reaction mixture during the course of protein expression, in order to extend reaction times. Alternatively, smaller initial concentrations followed by continuous or intermittent feeding may be employed.

[21] *In vitro* synthesis, as used herein, refers to the cell-free synthesis of biological macromolecules in a reaction mix comprising biological extracts and/or defined reagents. The reaction mix will comprise a template for production of the macromolecule, *e.g.* DNA, mRNA, *etc.*; monomers for the macromolecule to be synthesized, *e.g.* amino acids, nucleotides, *etc.*, and such co-factors, enzymes and other reagents that are necessary for the synthesis, *e.g.* ribosomes, tRNA, polymerases, transcriptional factors, *etc.* Such synthetic reaction systems are well-known in the art, and have been described in the literature. The cell free synthesis reaction may be performed as batch, continuous flow, or semi-continuous flow, as known in the art. The *in vitro* synthesis on biological macromolecules may include translation of mRNA to produce polypeptides or may include the transcription of mRNA from a DNA template.

[22] The reactions preferably utilize cell extracts derived from bacterial cells grown in medium containing glucose and phosphate, where the glucose is present at a concentration of at least about 0.25% (weight/volume), more usually at least about 1%; and usually not more than about 4%, more usually not more than about 2%. An example of such media is 2YTPG medium, however one of skill in the art will appreciate that many culture media can be adapted for this purpose, as there are many published media suitable for the growth of bacteria such as *E. coli*, using both defined and undefined sources of nutrients (see Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, 2nd edition. Cold Spring Harbor University Press, Cold Spring Harbor, NY for examples of glucose containing media).

[23] While conventional reaction mixtures (for example, see Kim and Swartz, 2001) contain about 2% polyethylene glycol 8000, it is found that this diminishes the yield. In the present methods, the molecules spermidine and putrescine are used in the place of PEG. Spermine or spermidine is present at a concentration of at least about 0.5 mM, usually at least about 1 mM, preferably about 1.5 mM, and not more than about 2.5 mM. Putrescine is present at a concentration of at least about 0.5 mM, preferably at least about 1 mM, preferably about 1.5 mM, and not more than about 2.5 mM.

[24] The concentration of magnesium in the reaction mixture affects the overall synthesis. Often there is magnesium present in the cell extracts, which may then be adjusted with

additional magnesium to optimize the concentration. Sources of magnesium salts useful in such methods are known in the art. In one embodiment of the invention, the source of magnesium is magnesium glutamate. A preferred concentration of magnesium is at least about 5 mM, usually at least about 10 mM, and preferably a least about 12 mM; and at a concentration of not more than about 20 mM, usually not more than about 15 mM.

[25] The system can be run under aerobic and anaerobic conditions. Oxygen may be supplied, particularly for reactions larger than 15μl, in order to increase synthesis yields. The headspace of the reaction chamber can be filled with oxygen; oxygen may be infused into the reaction mixture; *etc.* Oxygen can be supplied continuously or the headspace of the reaction chamber can be refilled during the course of protein expression for longer reaction times. Other electron acceptors, such as nitrate, sulfate, or fumarate may also be supplied in conjunction with preparing cell extracts so that the required enzymes are active in the cell extract.

[26] It is not necessary to add exogenous cofactors. Compounds such as nicotinamide adenine dinucleotide (NADH), NAD^+, or Coenzyme A may be used to supplement protein synthesis yields but are not required.

[27] The template for cell-free protein synthesis is preferably DNA. Coupled transcription and translation, generally utilized in *E. coli* systems, continuously generates mRNA from a DNA template with a recognizable promoter. Either endogenous RNA polymerase is used, or an exogenous phage RNA polymerase, typically T7 or SP6, is added directly to the reaction mixture. Alternatively, mRNA can be continually amplified by inserting the message into a template for QB replicase, an RNA dependent RNA polymerase. Nucleases can be removed from extracts to help stabilize mRNA levels. The template can encode for any particular gene of interest.

[28] Other salts, particularly those that are biologically relevant, such as manganese, may also be added. Potassium is generally present at a concentration of at least about 50 mM, and not more than about 250 mM. Ammonium may be present, usually at a concentration of not more than 200 mM, more usually at a concentration of not more than about 100 mM, and preferably at a concentration of not more than about 20 mM. Usually, the reaction is maintained in the range of about pH 5-10 and a temperature of about 20°-50° C; more usually, in the range of about pH 6-9 and a temperature of about 25°-40° C. These ranges may be extended for specific conditions of interest.

[29] Metabolic inhibitors to undesirable enzymatic activity may be added to the reaction mixture. Alternatively, enzymes or factors that are responsible for undesirable activity may be removed directly from the extract or the gene encoding the undesirable enzyme may be inactivated or deleted from the chromosome.

[30] Vesicles, either purified from the host organism (see Muller and Blobel (1984) "In vitro translocation of bacterial proteins across the plasma membrane of *Escherichia coli*", PNAS 81:7421-7425) or synthetic, may also be added to the system. These may be used to enhance protein synthesis and folding. The technology described herein has been shown to activate the oxidative phosphorylation process that utilizes cytoplasmic membrane components. Inverted membrane vesicles containing respiratory chain components and the F_1F_OATPase must be present for the activation of oxidative phosphorylation. The present methods also may be used for cell-free reactions to activate other sets of membrane proteins; for example, to insert or translocate proteins or to translocate other compounds.

METHODS FOR ENHANCED *IN VITRO* SYNTHESIS

[31] Synthetic systems of interest include systems for the replication of biopolymers, which can include amplification of DNA, transcription of RNA from DNA or RNA templates, translation of RNA into polypeptides, and the synthesis of complex carbohydrates from simple sugars. Enhanced synthesis may include increases in the total or relative amount of polypeptide synthesized in the system; increases in the total or relative amount of polypeptide synthesized per unit of time; increases in the total or relative amount of biologically active polypeptide synthesized in the system; increases in the total or relative amount of soluble polypeptide synthesized in the system, and the like.

[32] The reactions may utilize a large scale reactor, small scale, or may be multiplexed to perform a plurality of simultaneous syntheses. Continuous reactions will use a feed mechanism to introduce a flow of reagents, and may isolate the end-product as part of the process. Batch systems are also of interest, where additional reagents may be introduced to prolong the period of time for active synthesis. A reactor may be run in any mode such as batch, extended batch, semi-batch, semi-continuous, fed-batch and continuous, and which will be selected in accordance with the application purpose.

[33] The reactions may be of any volume, either in a small scale, usually at least about 1 μl and not more than about 15 μl, or in a scaled up reaction, where the reaction volume is at least about 15 μl, usually at least about 50 μl, more usually at least about 100 μl, and may be

500 µl, 1000 µl, or greater. In most cases, individual reactions will not be more than about 10 ml, although multiple reactions can be run in parallel. However, in principle, reactions may be conducted at any scale as long as sufficient oxygen (or other electron acceptor) is supplied.

[34] Of particular interest is the translation of mRNA to produce proteins, which translation may be combined with *in vitro* synthesis of mRNA from a DNA template. Such a cell-free system will contain all factors required for the translation of mRNA, for example ribosomes, amino acids, tRNAs, aminoacyl synthetases, elongation factors, initiation factors, and ribosome recycling factors. Cell-free systems known in the art include *E. coli* extracts, *etc.*, which can be treated with a suitable nuclease to eliminate active endogenous mRNA.

[35] In addition to the above components such as cell-free extract, genetic template, and amino acids, materials specifically required for protein synthesis may be added to the reaction. These materials include salt, folinic acid, cyclic AMP, inhibitors for protein or nucleic acid degrading enzymes, inhibitors or regulators of protein synthesis, adjusters of oxidation/reduction potential(s), non-denaturing surfactants, buffer components, spermine, spermidine, putrescine, *etc.*

[36] The salts preferably include potassium, magnesium, and ammonium salts (*e.g.* of acetic acid or sulfuric acid). One or more of such salts may have amino acids as a counter anion. There is an interdependence among ionic species for optimal concentration. These ionic species are typically optimized with regard to protein production. When changing the concentration of a particular component of the reaction medium, that of another component may be changed accordingly. For example, the concentrations of several components such as nucleotides and energy source compounds may be simultaneously controlled in accordance with the change in those of other components. Also, the concentration levels of components in the reactor may be varied over time. The adjuster of oxidation/reduction potential may be dithiothreitol, ascorbic acid, glutathione and/or their oxidized forms. Also, a non-denaturing surfactant such as Triton X-100 is optionally included, at a concentration of not more than about 500 mM, more usually not more than about 250 mM.

[37] When using a protein isolating means in a continuous operation mode, the product output from the reactor flows through a membrane and into the protein isolating means. In a semi-continuous operation mode, the outside or outer surface of the membrane is put into contact with predetermined solutions that are cyclically changed in a predetermined order. These solutions contain substrates such as amino acids and nucleotides. At this time, the

reactor is operated in dialysis, diafiltration batch or fed-batch mode. A feed solution may be supplied to the reactor through the same membrane or a separate injection unit. Synthesized protein is accumulated in the reactor, and then is isolated and purified according to the usual method for protein purification after completion of the system operation.

[38] Where there is a flow of reagents, the direction of liquid flow can be perpendicular and/or tangential to a membrane. Tangential flow is effective for recycling ATP and for preventing membrane plugging and may be superimposed on perpendicular flow. Flow perpendicular to the membrane may be caused or effected by a positive pressure pump or a vacuum suction pump. The solution in contact with the outside surface of the membrane may be cyclically changed, and may be in a steady tangential flow with respect to the membrane. The reactor may be stirred internally or externally by proper agitation means.

[39] During protein synthesis in the reactor, the protein isolating means for selectively isolating the desired protein may include a unit packed with particles coated with antibody molecules or other molecules immobilized with a component for adsorbing the synthesized, desired protein, and a membrane with pores of proper sizes. Preferably, the protein isolating means comprises two columns for alternating use.

[40] The amount of protein produced in a translation reaction can be measured in various fashions. One method relies on the availability of an assay which measures the activity of the particular protein being translated. An example of an assay for measuring protein activity is a luciferase assay system, or chloramphenical acetyl transferase assay system. These assays measure the amount of functionally active protein produced from the translation reaction. Activity assays will not measure full length protein that is inactive due to improper protein folding or lack of other post translational modifications necessary for protein activity.

[41] Another method of measuring the amount of protein produced in coupled in vitro transcription and translation reactions is to perform the reactions using a known quantity of radiolabeled amino acid such as ^{35}S-methionine, ^{3}H-leucine or ^{14}C-leucine and subsequently measuring the amount of radiolabeled amino acid incorporated into the newly translated protein. Incorporation assays will measure the amount of radiolabeled amino acids in all proteins produced in an *in vitro* translation reaction including truncated protein products. The radiolabeled protein may be further separated on a protein gel, and by autoradiography confirmed that the product is the proper size and that secondary protein products have not been produced.

[42] It is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, constructs, and reagents described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which will be limited only by the appended claims.

[43] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

[44] All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing, for example, the cell lines, constructs, and methodologies that are described in the publications, which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

[45] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the subject invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to ensure accuracy with respect to the numbers used (*e.g.* amounts, temperature, concentrations, *etc.*) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees centigrade; and pressure is at or near atmospheric.

EXPERIMENTAL

Methods and Materials

[46] The standard Cytomim environment for synthesis, contains the following components: 1.2 mM ATP, 0.85 mM each of GTP, UTP and CTP, 1 mM DTT, 130 mM potassium glutamate, 10 mM ammonium glutamate, 8 mM magnesium acetate, 34μg/ml folinic acid, 170.6 μg/ml *E. coli* tRNA mixture, 13.3 μg/ml plasmid, 100 μg/ml T7 RNA polymerase, 2 mM each of 20 unlabeled amino acids, 11 μM. [^{14}C]leucine, 1.5 mM spermidine and 1 mM

putrescine, 0.33 mM nicotinamide adenine dinucleotide, 0.26 mM Coenzyme A, 2.7 mM sodium oxalate and 0.24 volumes of S30 extract. Prokaryotic cell-free protein synthesis is performed using a crude S30 extract derived from *Escherichia coli* K12 (strain A19 ΔtonA ΔtnaA ΔspeA ΔendA ΔsdaA ΔsdaB met+), with slight modifications from the protocol of Pratt, J.M. 1984. (Coupled transcription-translation in prokaryotic cell-free systems. In Transcription and translation: a practical approach. Hanes, B.D., and S.J. Higgins. (Eds.). p. 179-209. IRL Press, New York.) Cells for the extract are grown with 2YTPG media (Kim and Choi). T7 RNA polymerase was prepared from *E. coli* strain BL21 (pAR1219) according to the procedures of Davanloo *et al.* 1984 (Cloning and expression of the gene for bacteriophage T7 RNA polymerase. Proc Nat'l Acad. Sci. USA 81:2035-2039.) The system can be enhanced with the addition of 33 mM sodium pyruvate, although this is not necessary. There was approximately an additional 3.3 mM magnesium, 14.4 mM potassium, 2.4 mM TRIS, and 23.5mM acetate in each reaction originating from the cell extract.

[47] The modified Cytomim environment for synthesis, according to the methods of the present invention, is as follows: 10 mM potassium phosphate and 50mM Bis-Tris (pH 7.0) was added, 5 μM of [^{14}C]leucine was used instead of 11μM. In addition, 30 mM glucose was added, 1.2 mM AMP was substituted for 1.2 mM ATP, and 0.85 mM each of GMP, UMP, and CMP were substituted for 0.85 mM each of GTP, UTP, and CTP. Additionally, oxalic acid was omitted.

[48] Reactions were incubated at 37°C for 3-6 hours. The amount of synthesized protein is estimated from the measured TCA-insoluble radioactivities using a liquid scintillation counter (Beckman LS3801). (Kim *et al.* 1996). Soluble protein yields were determined as previously described (Kim and Swartz 2000).

Results

[49] The standard reaction mixture for a coupled transcription-translation reaction is described by Kim and Swartz (2001). The energy source in the standard reaction is phosphoenolpyruvate (PEP). Previous direct substitution of PEP with glucose resulted in virtually no protein synthesis. However, when PEP was replaced by glucose-6-phosphate (G6P) significant protein yields of 228±13 μg/mL were observed (Kim and Swartz 2001). Glucose-6-phosphate is only one-step away from glucose in the glycolysis reaction pathway, suggesting that the initial phosphorylation step may be limiting. Several experiments were

conducted to alleviate this limitation including the addition of hexokinase or glucokinase to the reaction or slowly feeding glucose. Neither approach was successful.

[50] The G6P reaction continued to be optimized by modifications of the standard reaction mixture until protein yields using this energy source averaged over 700 μg/mL. To obtain these protein yields, a new pH buffer was used, and oxalic acid was removed. In addition, it was shown that NTPs could be replaced by NMPs in the G6P reaction giving similar yields. This substitution dramatically decreased the reaction cost. Despite these advances, the optimized conditions still did not allow significant protein synthesis when G6P was replaced with glucose.

[51] Glucose was successfully used as an energy source with NMPs when polyethylene glycol was replaced with the natural cations putrescine and spermidine and when additional phosphate was added to the optimized reaction. Phosphate is important both for the initial step of glycolysis (glucose to G6P) and for phosphorylation of the NMPs to NTPs. The cell-free reaction was phosphate-limited when using glucose as an energy source. This limitation did not exist in traditional cell-free reactions that typically utilize a phosphorylated energy source such as PEP, creatine phosphate, or even G6P. It was found that experiments using glucose plus phosphate gave protein yields of over 400 μg/mL, as compared to the experiments using G6P, which produced protein yields over 800 μg/mL. Glucose and NMP reactions are beneficial because of the decreased costs and increased stability of these reagents.

[52] Pyruvate, another nonphosphorylated energy source, was also used successfully in a cell-free protein synthesis reaction with NMPs only when additional phosphate was added. A three-hour batch reaction of the mixture described above was carried out after replacing glucose with pyruvate and NTPs with NMPs. Phosphate optimization resulted in protein yields that are close to the amount obtained for NTPs (Figure 1) at a fraction of the cost. Without any additional phosphate, protein synthesis yields are substantially lower.

[53] To further determine the requirements for this new reaction, reaction components were investigated individually. It was determined that reactions that use NTPs and glucose as an energy source also benefit from additional phosphate (Figure 2), resulting in similar yields to the NMP reaction. Further experiments were also conducted to determine other steps that are important for successful reactions. It was found that removal of PEG and oxalic acid, as well as careful pH control through the use of the appropriate buffer had a significant impact on protein yields (Figure 3). Previously, cell-free protein synthesis

reactions were limited to using phosphorylated energy sources and nucleotide triphosphates. These compounds are relatively expensive reagents in the cell-free reactions. In addition, the phosphorylated molecules are more susceptible to degradation and create a variable reaction environment with respect to inorganic phosphate concentration. Using glucose and nucleotide monophosphates will increase the reaction robustness and homeostasis while also dramatically decreasing costs. In fact, when the costs of energy sources and nucleotides are compared, glucose and NMPs are nearly two orders of magnitude less expensive than PEP and NTPs (Table 1, Row 1).

[54] Protein synthesis yields of *in vitro* synthesis reactions using glucose as an energy source and NMPs are approximately 60% of the traditional reaction. However, the great cost advantage of using glucose and NMPs compensates for the slight decrease in yield such that the relative product yield is almost ninety times better on a cost basis (Table 1, Row 3).

Table 1: Comparison of cell-free reactions with various energy sources and nucleotides

	NTPs			NMPs (+ phosphate)		
	PEP	G6P	Glucose	PEP	G6P	Glucose
Cost of energy source and nucleotides ($ / mL reaction)	1.72	0.71	0.56	1.18	0.17	0.013
Typical yields (μg / mL)	700	803	429	700	959	469
Relative product yield (μg protein / $)	1	2.8	1.9	1.5	14.3	87.5

WHAT IS CLAIMED IS:

1. A method for enhanced synthesis of biological macromolecules *in vitro*, the method comprising:

synthesizing said biological macromolecules in a reaction mix including a phosphate-free energy source, in the presence of exogenous phosphate.

2. The method of Claim 1, wherein said phosphate free energy source is glucose and/or pyruvate.

3. The method of Claim 2, wherein said phosphate is present at a concentration of from about 1 mM to about 40 mM.

4. The method of Claim 3, wherein said phosphate is provided as potassium phosphate, magnesium phosphate, and ammonium phosphate.

5. The method according to Claim 1, further comprising nucleoside monophosphates.

6. The method of Claim 5, wherein said synthesis of biological macromolecules comprises translation of mRNA to produce polypeptides.

7. The method of Claim 6, wherein said synthesis also comprises transcription of mRNA from a DNA template.

8. The method of Claim 1 wherein said synthesis of biological macromolecules is performed as a batch reaction.

9. The method of Claim 1, wherein said synthesis of biological macromolecules is performed as a continuous reaction.

10. The method of Claim 1, wherein said reaction mix comprises an extract from *E. coli* grown in glucose containing medium.

11. The method of Claim 10, wherein said *E. coli* are grown in glucose and phosphate containing medium.

12. The method of Claim 1, wherein said reaction mix comprises magnesium at a concentration of from about 5 mM to about 20 mM.

13. The method of Claim 1, wherein said reaction mix is substantially free of polyethylene glycol.

14. The method according to Claim 13, wherein said reaction mix comprises one or more of spermine, spermidine and putrescine.

IMPROVED METHODS OF *IN VITRO* PROTEIN SYNTHESIS

ABSTRACT OF THE INVENTION

[55] Improved methods are provided *in vitro* synthesis of biological molecules, providing for improved yields, lowered costs, and enhanced utility. Improved yield and lowered cost is obtained by the use of glucose as an energy source in the presence of exogenous phosphate.

Figure 1: Prot in produced in cell-fr e reactions using pyruvat as an energy source with NMPs and additional phosphat .

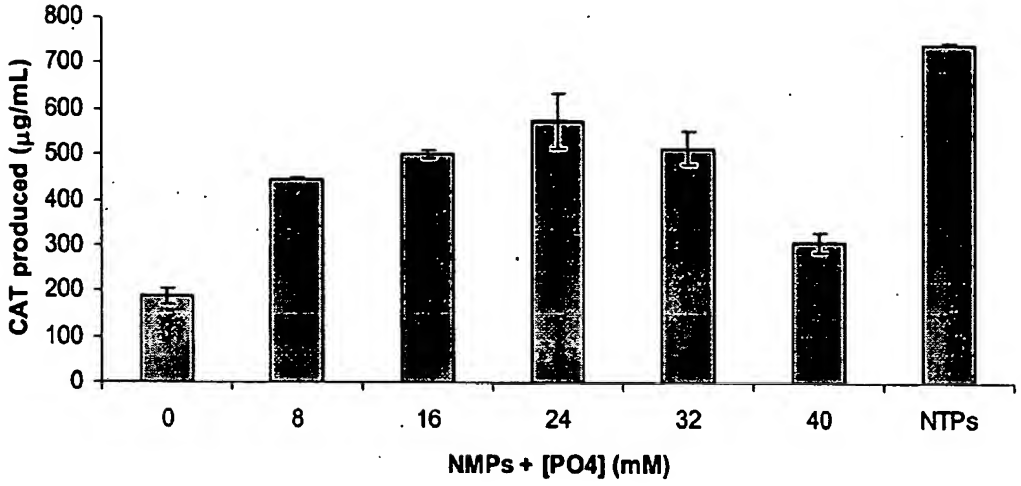
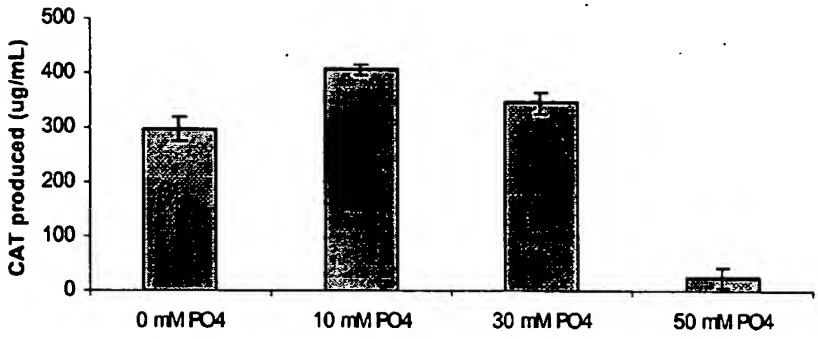
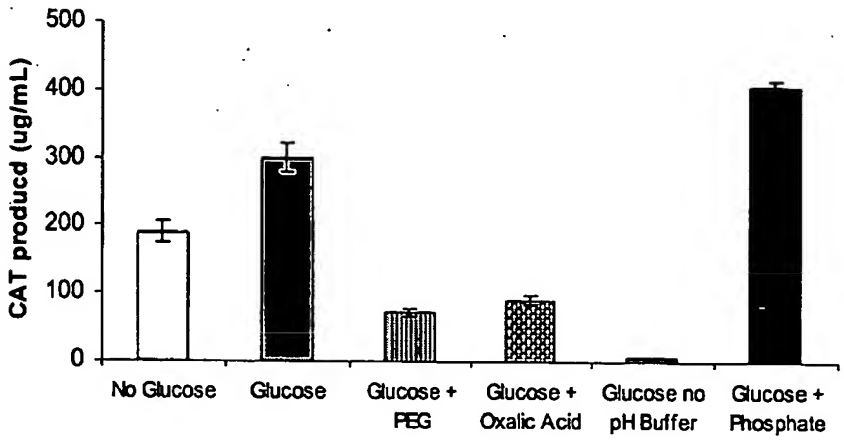


Figure 2: Addition of phosphate to cell-free reactions using glucose as energy source.



Figur 3: Important components for succ ssful cell-free r actions using glucose



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INVENTOR INFORMATION

Inventor One Given Name::	KARA
Family Name::	CALHOUN
Postal Address Line One::	Stanford University
Postal Address Line Two::	MC 5025
City::	Stanford
State or Province::	CA
Postal or Zip Code::	94305
Citizenship Country::	

Inventor Two Given Name::	JAMES ROBERT
Family Name::	SWARTZ
Postal Address Line One::	Dept Chem Eng
Postal Address Line Two:	Stauffer III
City::	Room 113
State or Province::	Stanford, California
Postal or Zip Code::	94305-5025
Citizenship Country::	US

CORRESPONDENCE INFORMATION

Correspondence Customer Number::	24353
Telephone One::	(650) 327-3400
Telephone Two::	(650) 833-7790
Fax::	(650) 327-3231
Electronic Mail::	sherwood@bozpat.com

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